INFECTION AND IMMUNITY, Nov. 1994, p. 5085–5094
0019-9567/94/$04.00+0

Vol. 62, No. 11

Purified Shiga-Like Toxins Induce Expression of Proinflammatory Cytokines from Murine Peritoneal Macrophages

VERNON L. TESH,[1]* BELAKERE RAMEGOWDA,[1] AND JAMES E. SAMUEL[1,2]

Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center, College Station, Texas 77843-1114,[1] and MicroCarb, Inc., Gaithersburg, Maryland 20879[2]

Received 13 April 1994/Returned for modification 22 June 1994/Accepted 18 August 1994

Infections with Shiga toxin-producing *Shigella dysenteriae* type 1 and Shiga-like toxin (SLT)-producing *Escherichia coli* cause outbreaks of bloody diarrhea in which patients are at risk for developing life-threatening complications involving the renal and central nervous systems. Histopathology studies and in vitro experiments suggested that the toxins damage toxin receptor-expressing endothelial cells (EC) lining glomerular and central nervous system capillaries. In the presence of inducible host factors (cytokines), EC sensitivity to SLT toxicity was increased ~1 million-fold. We hypothesized that to manifest the vascular lesions characteristic of infection with toxin-producing bacteria, two signals were needed: systemic toxins and elevated proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin 1 [IL-1], and IL-6). Human EC do not secrete these cytokines when stimulated with SLTs in vitro, suggesting that additional cells may be involved in pathogenesis. Therefore, we carried out comparative analyses of the capacity of purified (endotoxin-free) SLTs and lipopolysaccharides (LPS) to induce cytokine mRNA and proteins from murine macrophages. The cells were essentially refractory to SLT cytotoxicity, expressing low to undetectable levels of toxin receptor. SLTs and LPS induced TNF activity and IL-6 expression from macrophages, although dose response and kinetics of cytokine induction differed. LPS was a more effective inducing agent than SLTs. SLT-I-induced TNF activity and IL-6 expression were delayed compared with induction mediated by LPS. IL-1α production required ~24 h of exposure to SLTs or LPS. Macrophages from LPS-hyporesponsive C3H/HeJ mice produced low levels of TNF activity when treated with SLT-I, suggesting that LPS and SLTs may utilize separate signaling pathways for cytokine induction.

Infections with *Shigella dysenteriae* type 1 or enterohemorrhagic *Escherichia coli* are associated with outbreaks of bacillary dysentery or hemorrhagic colitis, respectively. Patients with these bloody diarrheal diseases are also at increased risk for the development of life-threatening complications involving the renal and central nervous systems (2, 27). One of these sequelae, the hemolytic-uremic syndrome (HUS), is a major cause of pediatric acute renal failure. *S. dysenteriae* type 1 expresses high levels of a potent cytotoxin called Shiga toxin. Enterohemorrhagic *E. coli* strains produce one or more exotoxins which are collectively referred to as Shiga-like toxins (SLTs) or verocytotoxins. Serologic and nucleotide sequencing analyses showed that SLTs may be classified on the basis of their antigenic and genetic relationship to Shiga toxin (reviewed in reference 42); SLT type I (SLT-I) is virtually identical to Shiga toxin, while SLT-II is ~56% homologous to Shiga toxin or SLT-I at the deduced amino acid sequence level. Although genetic variants of the toxins have been described, extensive biochemical and biologic studies have shown that all members of the SLT family are AB_5 holotoxins which act by inhibiting eukaryotic protein synthesis and utilize the neutral glycolipids globotriaosylceramide (Gb_3) or globotetraosylceramide (Gb_4) as functional receptors (reviewed in references 10, 35, and 42).

The precise role of the toxins in colonic ulceration and bloody diarrhea or in the development of postdiarrheal systemic complications is not known. Comparative histopathologic examination of tissues from macaque monkeys fed either an invasive toxigenic *S. dysenteriae* type 1 strain or an atoxigenic isogenic derivative suggested that Shiga toxin may exacerbate disease by specifically damaging capillaries serving the colonic mucosa and by mediating the influx of inflammatory cells into the intestinal compartment (17). Examination of kidney tissue from HUS patients showed damage to glomeruli, characterized by the swelling and detachment of glomerular endothelial cells (EC) from the basement membrane accompanied by the deposition of microthrombi and influx of inflammatory cells into the glomeruli (46, 47). Rabbits given intravenous SLTs developed severe edema and petechial hemorrhages in the cecal mucosa and widespread thrombotic microangiopathy, edema, and ischemic necrosis in the brain and spinal cord. Macrophages were occasionally found in the damaged tissues (4, 48, 65). Mice given SLTs developed acute bilateral renal tubular necrosis, and the sites of toxin-mediated damage and expression of Gb_3 were directly correlated (55, 61). Taken together, these data support the concept that the toxins act in vivo to specifically damage Gb_3-expressing cells in target organs. EC damage may then facilitate the exudation of neutrophils and monocytes into the affected tissues.

Shiga toxin and SLTs were shown to be directly toxic for human vascular EC in vitro (43, 58), although the doses of toxin necessary to manifest EC killing were 10^5 to 10^6 times greater than the doses used to lyse toxin-sensitive cell lines such as Vero or HeLa cells. We and others have found, however, that when EC were incubated in the presence of bacterial endotoxins or the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), they were markedly sensitized to the cytotoxic action of SLTs (33, 34, 58). The mechanism of cytokine-mediated toxin sensitization may involve increased synthesis and membrane expression of Gb_3 (59). Barrett et al. (3) demonstrated that TNF activity could be elicited from murine macrophages by treatment with purified

* Corresponding author. Phone: (409) 845-1313. Fax: (409) 845-3479. Electronic mail address: vltesh@sigma.tamu.edu.

SLT-II. On the basis of the collective results of these in vitro studies, we hypothesized that an immunologic component may be operative in the pathogenesis of dysenteric disease and the systemic sequelae associated with infection with SLT-producing bacteria (56). In addition to their pleiotropic systemic effects, cytokines produced by inflammatory cells in response to SLTs or endotoxin may act locally to exacerbate EC damage which is characteristic of *S. dysenteriae* type 1 or enterohemorrhagic *E. coli* infections. To test this hypothesis, we have carried out comparative analyses of the capacity of purified SLTs or lipopolysaccharide (LPS) to elicit proinflammatory cytokine proteins and TNF-α mRNA from macrophages stimulated in vitro. In comparison with toxin-sensitive Vero cells, thioglycolate-elicited murine peritoneal macrophages were relatively refractory to the cytotoxic action of SLTs. Despite the fact that murine macrophages expressed low to undetectable levels of the toxin receptor Gb_3, SLTs were effective inducers of TNF and IL-6 expression, but elevated levels of immunoreactive IL-1α were detectable only after prolonged exposure of the cells to the toxins. In comparison with SLTs, equivalent doses of LPS induced significantly higher levels of all three cytokines. The kinetics of cytokine production in response to treatment with SLTs or LPS also differed. The expression of elevated levels of TNF-α and IL-6 by macrophages in response to treatment with SLTs was consistently delayed compared with the response mediated by treatment with LPS. Macrophages derived from the LPS-hyporesponsive mouse strain C3H/HeJ produced soluble TNF when stimulated with purified SLTs but not when stimulated with LPS. Finally, pretreatment of LPS with polymyxin B reduced the ability of LPS to elicit TNF production but failed to affect TNF production induced by SLTs. These data suggest that SLTs and LPS may utilize separate signaling pathways to induce cytokine expression in murine macrophages.

MATERIALS AND METHODS

Toxin preparations. SLT-I and SLT-II were expressed from *Escherichia coli* DH5α transformed with plasmids containing the toxin operons under control of the T7 promoter. The *slt-I* operon on a 3.5-kb *Eco*RI-*Eco*RV fragment was subcloned from pJN25 (41) into the *Eco*RI-*Hin*cIII site of pBluescript SK^- (Stratagene, LaJolla, Calif.) and designated pCKS112 (gift of Clare Schmitt, Uniformed Services University of the Health Sciences). The *slt-II* operon was derived from plasmid pLMS2.2 (53). Subsequently, the recombinant strains were transformed with the plasmid pGP1-2 containing the thermoinducible T7 polymerase gene (54). These strains were maintained under BL3+EK1 containment (15). SLT-I and SLT-II in crude bacterial lysates were purified by sequential ion-exchange, chromatofocusing, and immunoaffinity chromatography as described previously (49, 55). Toxins were assessed for homogeneity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with silver staining and Western blots (immunoblots). Toxin preparations were passed through ActiClean Etox columns (Sterogene Bioseparations, Arcadia, Calif.) to remove trace endotoxin contaminants and contained <0.1 ng of endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay. Purified LPS derived from *E. coli* O111:B4 was purchased from Sigma Chemical Co., St. Louis, Mo.

Mice. C3H/HeN (lps^n/lps^n) mice were purchased from Taconic Farms, Germantown, N.Y. C3H/HeJ (lps^d/lps^d) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were 4 to 8 weeks old when used in these experiments. Mice were housed in single-vendor laminar-flow facilities on a 12-h light–12 h dark cycle and given access to water and chow ad libitum throughout the course of the experiments. The experiments performed here were conducted according to principles set forth in the *Guide for the Care and Use of Laboratory Animals* (40).

Cell culture and stimulation. Mice were injected intraperitoneally with sterile 3% thioglycolate. Four days later, peritoneal exudative cells were collected by lavage with Hanks' balanced salt solution containing heparin (350 μg/ml). Cell suspensions were adjusted to 10^6 cells per ml in Eagle's minimum essential medium (EMEM; Gibco, Madison, Wis.) containing penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and 5% fetal bovine serum (Intergen, Purchase, N.Y.) in sterile culture dishes (35 by 10 mm; Becton Dickinson Labware, Oxnard, Calif.). After 2 h of incubation at 37°C in humidified 5% CO_2, each culture dish was washed twice with fresh EMEM to remove nonadherent cells. After overnight incubation, the medium was replaced with fresh complete EMEM containing various doses of purified SLTs or LPS and incubated at 37°C in 5% CO_2 for the time periods indicated in the text. In some experiments, macrophages were stimulated with SLTs or LPS which had been preincubated at room temperature with 20 μg of polymyxin B (Sigma) per ml for 6 h prior to addition to the cells. At each time point, culture supernatants and cells were collected for cytokine mRNA and protein quantitation. Vero cells were purchased from the American Type Culture Collection, Rockville, Md., and maintained in EMEM plus 5% fetal bovine serum. Human umbilical vein EC (gift of David J. Silverman, University of Maryland School of Medicine) were derived and cultured as described by Santucci et al. (51).

Cytotoxicity assay. Murine peritoneal macrophages (~10^5 cells per well) or Vero cells (~10^4 cells per well) were cultured in 96-well microtiter plates for 24 h at 37°C in humidified CO_2. Toxin dilutions were prepared in complete EMEM, and 100 μl of each dilution was added to six separate wells. Cells were incubated with toxins for 18, 36, and 48 h, at which time the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) (39) was added and incubation was continued for another 2 h. The cells were lysed, and the insoluble formazan dye was extracted for 4 h at 37°C with lysis buffer (50% *N,N*-dimethyl formamide and 20% SDS [pH 4.7]). The A_{570} was measured. Untreated macrophages and Vero cells were used as viability controls, and blank wells were used to determine background absorbance. Percentage viability was calculated as [(A_{570} of treated cells − background)/(A_{570} of untreated cells − background)] × 100.

Membrane glycolipid quantitation. Total lipid extracts from murine macrophages, Vero cells, and human EC as well as purified glycolipid controls were subjected to chromatography on aluminum-backed silica thin-layer chromatography plates (Silica Gel 60; Merck AG, Darmstadt, Germany) with $CHCl_3$-CH_3OH-aqueous KCl (5:4:1) as described previously (49). Following chromatographic separation of the glycolipids, the plates were coated with 0.1% polyisobutylmethacrylate (Polyscience, Warrington, Pa.), air dried, and sprayed with Tris-buffered saline–bovine serum albumin (TBS-BSA; 0.1 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1% BSA). The plates were treated with purified SLT-I in TBS-BSA at 4°C for 18 h. After extensive washing, the plates were sequentially treated with a monoclonal antibody directed against SLT-I B subunits (monoclonal antibody 13C4) (52) and ^{125}I-labeled goat anti-mouse immunoglobulin G secondary antibody. Specific toxin binding to glycolipid bands was visualized by autoradiography. Relative amounts of toxin-binding glycolipids in the extracts were estimated by comparison with serial dilutions of purified

Gb_3 separated on the same plates by densitometric scanning of the films (IS-1000 Digital Imaging System with IA-860 Imaging Analysis software; Innotech Corp., San Leandro, Calif.).

Cytokine assays. TNF bioactivity in macrophage supernatants was assessed by measuring lysis of L929 murine fibroblasts by the procedure described by Hill and McCallum (22). Briefly, L929 cells were cultured in 96-well microtiter plates at a density of 2×10^5 cells per well in Iscove's modified Dulbecco medium (Mediatech, Washington, D.C.) supplemented with 5% fetal bovine serum. Dilutions of macrophage supernatants or recombinant murine TNF-α standard (Genentech, South San Francisco, Calif.) were made in medium containing 1 μg of actinomycin D (Sigma) per ml and added in quadruplicate to the cells. After 18 h of incubation at 37°C in humidified 5% CO_2, MTT was added and incubation was continued for another 2 h. The cells were lysed, and the formazan dye was extracted for 4 h at 37°C with 50% *N,N*-dimethyl formamide and 20% SDS. The A_{570} was measured. The amount of TNF present in the samples was estimated by comparison of the survival values with a recombinant murine TNF-α standard curve generated by linear regression analysis. Controls included L929 cells treated with actinomycin D alone and cells treated with actinomycin D plus SLT-I (100 ng/ml). Compared with untreated cells, L929 cytotoxicity mediated by actinomycin D plus SLT-I was consistently <10%. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used as described in the manufacturer's instructions to quantitate levels of immunoreactive murine IL-1α (Genzyme Corp., Boston, Mass.) and IL-6 (Endogen Inc., Boston, Mass.). Statistical analyses using a paired *t* test were performed with Microsoft Excel version 4.0 software (Microsoft Corp., Redmond, Wash.).

Preparation and labeling of murine TNF-α and GAPDH nucleotide probes. By using an automated DNA synthesizer (Applied Biosystems, Foster City, Calif.), the following sense and antisense oligonucleotide primers were synthesized: 5′-CCCCAAAAGATGGGGGGC-3′ and 5′-CCCATCGGCTG GCACACAC-3′, corresponding to nucleotides 221 to 238 and 568 to 551 of the murine TNF-α cDNA (11), and 5′-CCATG GAGAAGGCTGGG-3′ and 5′-CAAAGTTGTCATGGAT GACC-3′, corresponding to nucleotides 386 to 403 and 580 to 561 of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (18). The respective primers were used to amplify a 345-bp fragment of murine TNF-α cDNA or a 195-bp fragment of GAPDH cDNA by PCR. Each deoxynucleoside triphosphate (0.2 μM) and a 0.4 μM concentration of each of the sense and antisense primers were added to PCR buffer (10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin). The amplification profile was: denaturation, 95°C, 1 min; annealing, 55°C, 1 min; extension, 72°C, 2 min, for 30 cycles followed by an extension incubation at 72°C for 7 min. PCR products were extracted with phenol-chloroform and precipitated with ethanol and ammonium acetate. Aliquots of the PCR products were electrophoresed into 2% agarose gels and stained with ethidium bromide, and the electrophoretic migration was compared with that of the DNA standard prepared from *Hae*III-digested ϕX174 DNA. PCR product purity was assessed by measuring optical densities at 260 and 280 nm. Approximately 25-ng samples of the PCR products were then used as templates for radiolabeling by random priming with [^{32}P]dCTP (Prime-A-Gene kit; Amersham Corp., Arlington Heights, Ill.). The probes were purified by using Sephadex G-50 spin columns (5 Prime-3 Prime Inc., Boulder, Colo.).

RNA isolation and quantitation. Following stimulation for the indicated time periods, macrophage monolayers were detached by using sterile rubber policemen and the cells were pelleted by centrifugation at 735 × *g*. Cell pellets were lysed in ice-cold guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate [pH 5.2], 2% Sarkosyl, 1% mercaptoethanol) for 20 min. Nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ammonium acetate and ethanol. RNA purity was assessed by measuring optical densities at 260 and 280 nm. Three micrograms of RNA from each sample was applied under vacuum to nylon membranes (GeneScreen Plus; NEN Research Products, Boston, Mass.) by using a slot blot apparatus (Filtration Manifold System; Gibco/BRL, Gaithersburg, Md.). The blots were prehybridized in Rapid-hyb Buffer (Amersham Corp.) for 15 min at 65°C in a Hybridiser HB-1D hybridization oven (Techne Inc., Princeton, N.J.) and hybridized in buffer containing ~6 × 10^6 cpm of the TNF-α or GAPDH probe. After a 3-h incubation, the blots were washed at room temperature in 2× SSC–0.1× SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min with gentle agitation. The blots were dried, and the radioactivity associated with macrophage RNA was directly quantitated for 1 h with a Betascope 603 blot analyzer (Betagen Corp., Waltham, Mass.). The blots were then exposed to X-ray film for 2 to 4 days. Data are expressed as the ratio of TNF-α counts/GAPDH counts.

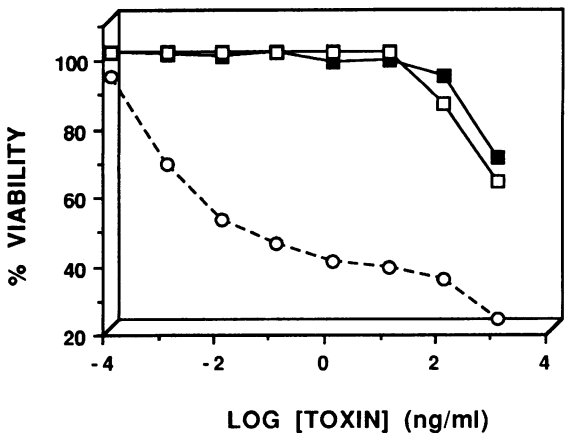

FIG. 1. Comparative cytotoxicities of SLT-I and SLT-II for murine macrophages and Vero cells. Cells were incubated with 10-fold dilutions of the toxins in six wells for 48 h at 37°C in humidified 5% CO_2. Viability was measured by uptake of the dye MTT. Untreated cells were used to determine percent viability. The data shown are the averages of 12 determinations from two separate experiments. Symbols: ○, cytotoxicity of SLT-I for Vero cells; ■, cytotoxicity of SLT-I for murine macrophages; □, cytotoxicity of SLT-II for murine macrophages.

RESULTS

Cytotoxicity studies. The cytotoxicity of SLT-I and SLT-II for murine peritoneal macrophages and Vero cells was assessed by incubating cells for 18, 36, and 48 h with serial 10-fold dilutions of the purified toxins. Cells were then treated with the dye MTT and lysed, and the optical density at 570 nm was measured. In accordance with earlier studies (28), Vero cells were sensitive to the cytotoxic action of SLT-I (Fig. 1) and SLT-II (data not shown); the toxin doses necessary to kill 50% of the cells in a well were ~1.0 to 10 pg. In contrast, murine peritoneal macrophages were generally refractory to the cytotoxic activity of the toxins at all of the time points tested; only incubation of macrophages for 48 h with the highest dose of

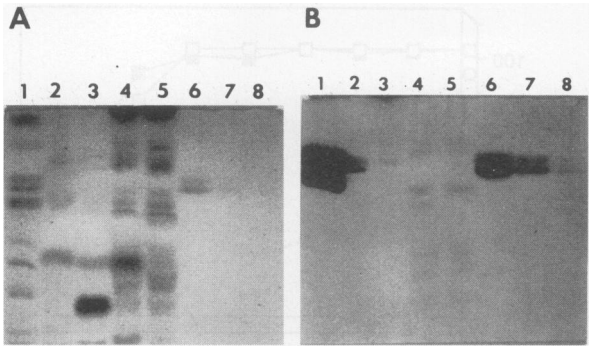

FIG. 2. Characterization of SLT-binding glycolipids derived from murine macrophages. (A) Orcinol-stained glycolipids; (B) glycolipids separated identically to those in panel A and subsequently treated with purified SLT-I, anti-SLT-I monoclonal antibody; and ^{125}I-labeled secondary antibody. Toxin binding was visualized by autoradiography. Lanes: 1, purified glycolipid standards; 2, glycolipids derived from Vero cells (1 mg); 3, human umbilical vein EC (3 mg); 4, C3H/HeJ macrophages (10 mg); 5, C3H/HeN macrophages (10 mg); 6, purified Gb_3 (1 μg); 7, purified Gb_3 (0.1 μg); 8, purified Gb_3 (0.01 μg). The film was intentionally overexposed to visualize the faint toxin-binding bands in macrophage extracts.

toxins tested (1.0 μg per well) manifested cytotoxicity. Macrophages, which have been demonstrated to be key cellular mediators of the immune response to bacteria and bacterial antigens, were not killed in vitro by treatment with SLT-I or SLT-II over a broad concentration range.

Quantitative analysis of toxin receptor expression in murine macrophages. Numerous studies have demonstrated that neutral glycolipids of the globo series, found in the membranes of a variety of mammalian cells, serve as toxin receptors and that the level of toxin receptor expression may be a critical determinant of susceptibility to cytotoxicity (10, 24, 35). Binding of the holotoxin to the glycolipid Gb_3 results in receptor-mediated endocytosis, intracellular trafficking of the toxin to the trans-Golgi network and endoplasmic reticulum, and, ultimately, the translocation of the enzymatic A subunit into the cytosol where ribosome inactivation occurs (50). Purified SLT-I and monoclonal antibody directed against SLT-I were used to detect and compare the levels of toxin-binding glycolipids present in murine macrophages, Vero cells (toxin-sensitive cells, high Gb_3 expression), and human umbilical vein EC (relatively toxin-insensitive cells, low Gb_3 expression). Total membrane glycolipid extracts and glycolipid standards were separated by thin-layer chromatography, and dilutions of purified Gb_3 separated on the same plates were used to quantitate glycolipids present in the membranes of each cell type (Fig. 2). In accordance with earlier studies (58), we estimated that Vero cells (Fig. 2, lane 2) and EC (lane 3) contained ~80 nmol of Gb_3 per mg of cells and 100 pmol of Gb_3 per mg of cells, respectively. Macrophages derived from the LPS-hyporesponsive C3H/HeJ mouse strain (lane 4) expressed undetectable levels of the toxin-binding glycolipid (<10 pmol of Gb_3 per mg of cells) in this assay. Macrophages derived from C3H/HeN mice (lane 5) contained barely detectable levels of Gb_3 (~20 pmol of Gb_3 per mg of cells). Both C3H/HeN and C3H/HeJ macrophages possessed an additional toxin-binding glycolipid which migrated with purified Gb_4. These data support the concept that the relative resistance of murine macrophages to the cytotoxic action of SLT-I and SLT-II may be due, in part, to low levels of Gb_3 in the macrophage membranes.

Kinetics of TNF production by murine macrophages stimulated with SLTs or LPS. We and others showed that pre- or coincubation of human venous EC with recombinant human TNF-α and purified Shiga toxin or SLTs sensitized the EC to cytotoxicity (33, 58, 59). Furthermore, human EC do not secrete elevated levels of TNF-α when stimulated with sublethal concentrations of purified Shiga toxin (57). These data suggest that although cytokines may upregulate the expression of Gb_3 by EC and participate in the development of vascular injury characteristic of HUS and other postdiarrheal sequelae, EC may not be the source of the cytokines. In light of the report by Barrett et al. (3) that macrophages respond to SLT-II with elevated TNF activity and histopathological studies showing an influx of inflammatory cells into sites of toxin-mediated damage (46–48), we examined whether macrophages produce TNF and other proinflammatory cytokines when stimulated with the toxins. It should also be noted that patients with bloody diarrhea and HUS are often endotoxemic (30). Unlike SLT-macrophage interactions leading to cytokine production, which have not been systematically examined, it is now well documented that LPS is a potent inducer of proinflammatory cytokine production from macrophages in vitro and systemically in vivo (38, 45). We therefore quantitated and compared cytokine production by macrophages derived from LPS-responsive and LPS-hyporesponsive mice in response to treatment with purified LPS or SLTs.

The kinetics of TNF production by murine macrophages in response to treatment with purified SLT-I or LPS was measured by the L929 cytotoxicity assay (Fig. 3). TNF activity was rapidly induced from C3H/HeN macrophages treated with LPS (10 ng/ml), showing a slight elevation at the 0.5- and 1-h time points and a major elevation at the 3-h time point. TNF activity remained elevated throughout the course of the experiment (36 h). As expected, macrophages derived from the C3H/HeJ mouse strain did not produce elevated levels of TNF activity following treatment with LPS. In contrast to the rapid induction of TNF activity from C3H/HeN macrophages by LPS, the induction of TNF activity by SLT-I was significantly delayed. After 9 h of stimulation with purified SLT-I (100 ng/ml), low levels of TNF activity (25 to 50 U/ml) were detected in C3H/HeN macrophage culture supernatants. After 12 h, TNF activity levels began to rise and reached near-maximal levels (~200 U/ml) by 15 h. TNF production in response to SLT-I then remained elevated throughout the course of the experiment but did not reach the same levels induced by a 10-fold-lower dose of LPS at any time point. In comparison with results of treatment with LPS, C3H/HeJ macrophages produced low levels of TNF activity (~50 U/ml) in response to stimulation with purified SLT-I ($P < 0.001$ by paired t tests).

The control of TNF synthesis and secretion is complex, involving regulation of transcriptional, translational, and post-translational processes. Beutler et al. (7) used nuclear transcription assays and Northern (RNA) blot analysis of mRNA extracted from C3H/HeN and C3H/HeJ macrophages to show that the LPS-hyporesponsive cells did not produce elevated levels of TNF mRNA in response to stimulation with 10 ng of LPS per ml. In conjunction with the experiments to characterize the induction of TNF activity in murine macrophages stimulated with SLT-I, we also extracted total cytoplasmic RNA from the cells at each time point to examine the kinetics of TNF-α mRNA induction. Equivalent amounts of RNA were blotted onto nylon membranes and hybridized to ^{32}P-labeled TNF-α (Fig. 4A) or GAPDH probes (Fig. 4B), and induction of TNF-α mRNA was compared with mRNA levels of the housekeeping gene (Fig. 4C). At the earliest time point tested (0.5 h), TNF-α transcript levels were elevated in SLT-I-treated

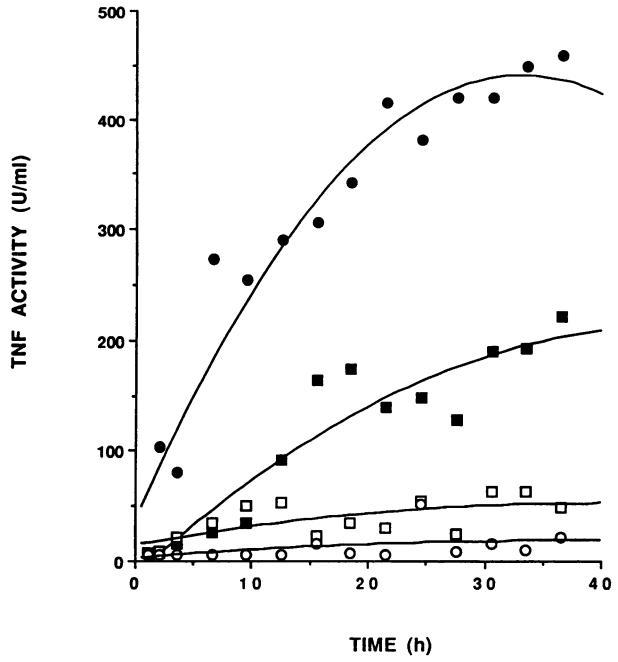

FIG. 3. Kinetics of induction of TNF activity by SLT-I- or LPS-treated peritoneal macrophages from C3H/HeN and C3H/HeJ mice. Approximately 10^6 cells per ml were stimulated with purified SLT-I (100 ng/ml) or LPS (10 ng/ml) and incubated at 37°C in humidified 5% CO_2. At the indicated time points, culture supernatants were collected and TNF activity was quantitated in quadruplicate samples by the L929 lysis assay described in Materials and Methods. The data shown are the averages of 12 determinations from three separate experiments. P was <0.001 when comparing SLT-I- and LPS-treated C3H/HeJ macrophages by a paired t test. Symbols: ●, LPS-treated macrophages from C3H/HeN mice; ■, SLT-I-treated macrophages from C3H/HeN mice; □, SLT-I-treated macrophages from C3H/HeJ mice; ○, LPS-treated macrophages from C3H/HeJ mice.

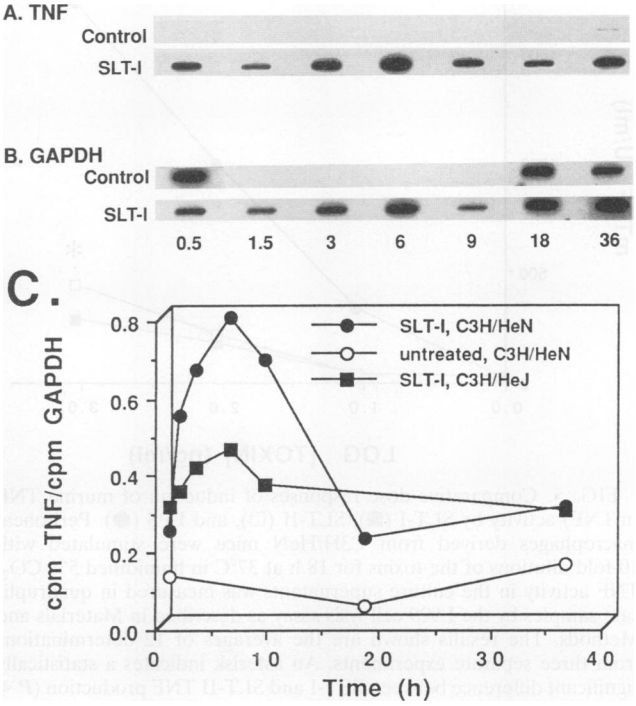

FIG. 4. Kinetics of TNF-α transcriptional activation in C3H/HeN and C3H/HeJ macrophages stimulated with SLT-I. Three micrograms of total cytoplasmic RNA extracted from untreated (control) C3H/HeN macrophages and from macrophages treated with purified SLT-I (100 ng/ml) for the indicated time points (0.5 to 36 h) were transferred to nylon filters and hybridized to ^{32}P-labeled cDNA probes specific for murine TNF-α (A) or GAPDH (B). Prior to exposing the blots to X-ray film, the radioactivity bound to the blots was directly counted for 1 h with a Betascope 603 blot analyzer, and the ratio of counts hybridizing to TNF-α to those of GAPDH mRNA was determined at each time point (C).

C3H/HeN macrophages, and the levels continued to rise until peaking at 6 h of stimulation. At the times of optimal soluble TNF production (12 to 15 h), the levels of TNF-α transcripts were diminished. This difference in kinetics of mRNA and protein production probably represents the time necessary for posttranslational processing and secretion of TNF. In contrast, treatment of C3H/HeJ macrophages with an equivalent dose of SLT-I induced much lower levels of TNF transcripts, although the kinetics of induction were similar to that seen in C3H/HeN cells. In accordance with earlier studies examining TNF mRNA induction in response to LPS (7, 62, 64, 66), we found that TNF-α mRNA levels were rapidly induced in C3H/HeN macrophages by treatment with LPS, peaking at ~1 h and rapidly declining thereafter (data not shown). Thus, the kinetic profiles of TNF production mediated by SLTs or LPS differ at both the transcriptional and translational or posttranslational levels.

SLT and LPS dose responses for murine macrophage TNF production. C3H/HeN macrophages were incubated with 10-fold dilutions of purified SLT-I, SLT-II, and LPS for 18 h, and TNF activity in culture supernatants was assessed by the L929 cytotoxicity assay (Fig. 5). We detected low levels of TNF activity in untreated cells. The SLTs were less effective than LPS at inducing TNF activity from murine macrophages; ~10-fold-higher doses of SLTs were necessary to induce equivalent TNF activities mediated by LPS. The dose responses for TNF production by SLT-I and SLT-II were not significantly different except at the highest toxin dose (1 μg/ml) tested.

The concentrated purified SLT preparations used in this study were nonreactive in the *Limulus* amoebocyte lysate assay and were subsequently diluted at least 40- to 50-fold in endotoxin-free media prior to addition to cells. However, earlier studies have shown that macrophages may be stimulated in vitro to produce TNF in response to picogram quantities of LPS (64). We wished, therefore, to ensure that the TNF activity we measured following treatment of macrophages with SLTs was not due to the presence of minute quantities of endotoxin contaminants in the toxin preparations. We assessed the capacity of macrophages derived from LPS-responsive C3H/HeN mice to express TNF activity following stimulation with SLT (100-ng/ml) or LPS (5-ng/ml) preparations which had been pretreated with the drug polymyxin B. Polymyxin B binds to the lipid A moiety of endotoxin and inhibits many of its endotoxic activities, including the ability to elicit TNF activity from macrophages at LPS doses up to ~10 ng/ml (29). Pretreatment of LPS with polymyxin B inhibited TNF-inducing activity, while the identical treatment of SLTs did not affect the subsequent stimulation of TNF activity from macrophages (Table 1). These data suggest that the reproducible induction of TNF activity from murine macrophages by treatment with SLTs is not due to minute quantities of contaminating endotoxin.

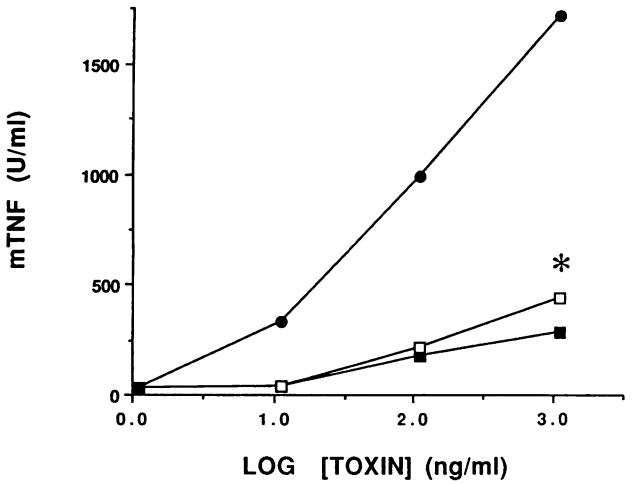

FIG. 5. Comparative dose responses of induction of murine TNF (mTNF) activity by SLT-I (■), SLT-II (□), and LPS (●). Peritoneal macrophages derived from C3H/HeN mice were stimulated with 10-fold dilutions of the toxins for 18 h at 37°C in humidified 5% CO_2. TNF activity in the culture supernatants was measured in quadruplicate samples by the L929 cell lysis assay as described in Materials and Methods. The results shown are the averages of 12 determinations from three separate experiments. An asterisk indicates a statistically significant difference between SLT-I and SLT-II TNF production ($P < 0.01$ by a paired t test).

Production of IL-1α and IL-6 by murine macrophages treated with SLTs. IL-1α and IL-6 are pleiotropic cytokines which profoundly alter immune responsiveness (reviewed in references 1 and 14). IL-1α acts on macrophages to induce its own synthesis and the synthesis of TNF-α and IL-6. A membrane-associated form of IL-1α may be important in focusing the immune response to sites of inflammatory cell infiltration. IL-6 acts on B lymphocytes to drive differentiation and antibody production. In the presence of comitogens, IL-6 acts on T cells to stimulate IL-2 and IL-2 receptor expression. IL-6 mediates increased hepatocyte acute-phase protein synthesis. Macrophages derived from C3H/HeN mice and treated with 100 ng of SLT-I per ml or 10 ng of LPS per ml produced elevated levels of soluble immunoreactive IL-1α after prolonged incubation (24 to 27 h) with the inducers (Fig. 6A). As was the case with TNF synthesis, we noted that IL-1α levels produced by LPS- and SLT-I-treated C3H/HeJ cells were reduced (~20 pg/ml at 36 h) compared with IL-1α levels produced by C3H/HeN cells. Both stimulants induced IL-6 expression from C3H/HeN cells, although the kinetics differed (Fig. 6B). LPS treatment resulted in elevated production and secretion of IL-6 beginning at approximately 6 h poststimulation. In contrast, elevated levels of IL-6 were first detected in macrophage supernatants after 12 h of stimulation with SLT-I.

TABLE 1. Polymyxin B pretreatment does not inhibit SLT-I-mediated TNF production by murine peritoneal macrophages

Treatment[a]	[muTNF][b]
None	<10
LPS (5 ng/ml)	4,600 ± 450
LPS + Poly B[c]	1,410 ± 20
SLT-I (100 ng/ml)	4,267 ± 272
SLT-I + Poly B	4,171 ± 124
Poly B	<10

[a] Peritoneal macrophages derived from C3H/HeN mice were treated with stimulants for 18 h at 37°C in 5% CO_2. Supernatants were collected, and soluble murine TNF (muTNF) activity was determined in quadruplicate by using the L929 cytotoxicity assay (see Materials and Methods). Results shown are the averages of eight determinations from two separate experiments.

[b] Concentration of soluble TNF in picograms per milliliter ± standard error of the mean.

[c] Poly B, stimulants were pretreated with 20 μg of polymyxin B for 6 h at room temperature prior to addition to the cells.

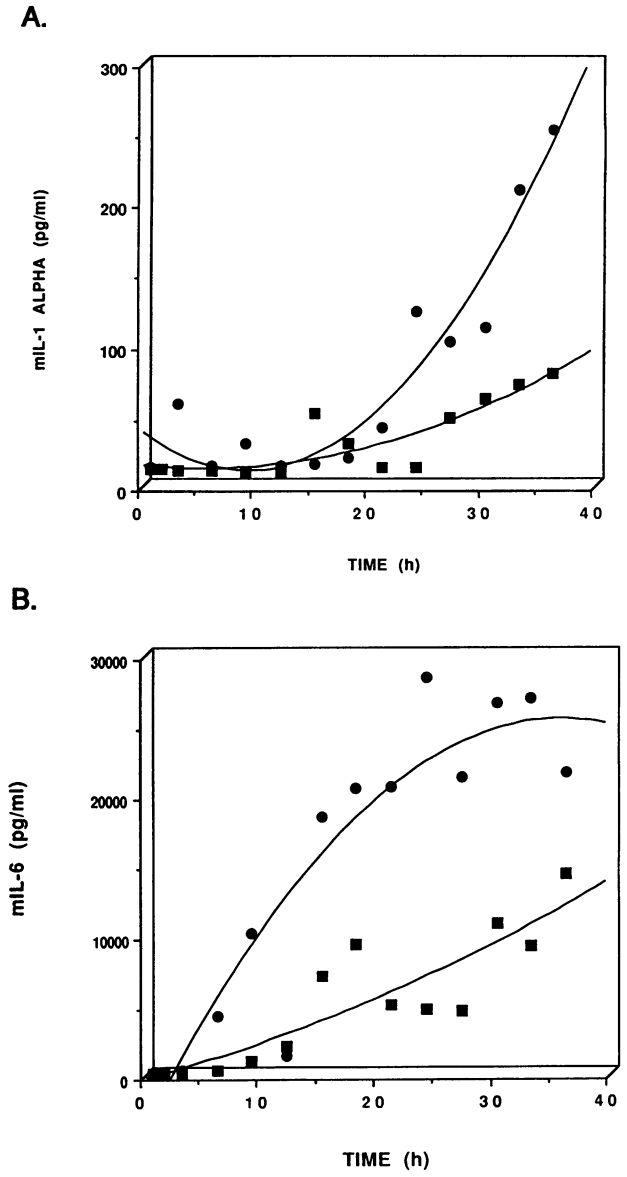

FIG. 6. Kinetics of IL-1α (A) and IL-6 (B) production by C3H/HeN macrophages stimulated by SLT-I (■) or LPS (●). Peritoneal macrophages were stimulated with SLT-I (100 ng/ml) or LPS (10 ng/ml) and incubated at 37°C in humidified 5% CO_2. At the indicated time points, culture supernatants were collected, and the levels of immunoreactive IL-1α (mIL-1 ALPHA) and IL-6 (mIL-6) were measured in triplicate by an ELISA. Results shown are the averages of six determinations from two separate experiments. Note the differences in y-axis scales.

DISCUSSION

Studies on the role of Shiga toxin and SLTs in pathogenesis have focused primarily on the potent cytotoxic activity of the toxins, and indeed, direct toxin-mediated cytotoxicity may be an important component in colonic ulceration and the subsequent vascular damage in the central nervous system and kidneys characteristic of human infection with SLT-producing bacteria. Recent studies have, however, documented the importance of proinflammatory cytokines in sensitizing Gb_3-expressing target cells to the cytopathic effect of SLTs (33, 34, 58, 59). A number of bacterial toxins, including *Bacillus anthracis* lethal toxin (19), *Clostridium difficile* toxins A and B (16), *E. coli* hemolysin (8), *Pseudomonas aeruginosa* exotoxin A (37), *Staphylococcus aureus* toxic shock syndrome toxin 1 (23, 44), and *Vibrio cholerae* cholera toxin (9), have been shown to induce cytokine expression from monocytes/macrophages. We have, therefore, focused on the interaction of SLTs with murine macrophages, cells which regulate immune responsiveness, in part, by producing multiple pleiotropic cytokines. Murine macrophages are approximately 10^5 to 10^6 times more refractory to the direct cytotoxic action of SLTs than toxin-sensitive cell lines primarily of epithelial cell origin. Earlier studies showed a direct correlation between levels of Gb_3 membrane expression and sensitivity to SLT cytotoxicity (24, 58). We show here that macrophage resistance to SLT toxic action is due, at least in part, to reduced levels of toxin receptor expression in macrophage membranes.

Although macrophages are not killed by treatment with physiologically relevant doses of the toxins, they do respond by producing elevated levels of the proinflammatory cytokines TNF-α, IL-1α, and IL-6. These findings are significant in light of earlier studies showing that human EC become exquisitively sensitive to the cytotoxic action of SLTs when coincubated with TNF-α or IL-1. We have shown that EC probably do not contribute to their own demise; that is, they do not produce elevated levels of TNF-α or IL-1 when stimulated with purified SLTs in vitro (57). However, the tissue damage seen in dysenteric patients, or which occurs after the administration of purified SLTs to animals, is frequently characterized by an inflammatory cell influx into the sites of damage. Thus, resident macrophage-like cells (e.g., mesangial cells or astrocytes) or infiltrating monocytic cells may contribute to the development of vascular lesions in glomeruli or central nervous system tissue by producing elevated levels of cytokines. In support of this hypothesis, Harel et al. (20) injected TNF-chloramphenicol acetyltransferase-transgenic mice with purified SLT-I and noted a marked induction of TNF which was specifically limited to the kidneys. Earlier studies demonstrated that toxin administration to mice resulted in tissue damage limited to renal cortical tubules (55, 61). Thus, the development of HUS and vascular lesions at other sites may involve multiple signals; tissues damaged by circulating SLTs may contain target cells expressing high levels of Gb_3 and resident or infiltrating cells which produce cytokines in response to the toxins.

In the studies reported here, we have used LPS, a potent cytokine-eliciting agent, for comparative purposes. Although the macrophage cytokine response to purified SLTs is less vigorous than that to LPS, it should be noted that *Shigella* spp. and enterohemorrhagic *E. coli* mediate considerable damage to the epithelial lining of the large intestine and patients with bloody diarrhea or HUS are frequently endotoxemic (30). In vitro studies have shown that astrocytes, mesangial cells, and renal tubular epithelial cells produce TNF in response to stimulation with LPS (5, 25, 32). It is not unreasonable, therefore, to speculate that multiple bacterial components may contribute to the development of EC damage seen in vivo. Studies designed to examine the capacity of SLTs and LPS to induce cytokines in an additive or synergistic fashion are currently under way.

At present, neither the holotoxin subunit(s) responsible for binding nor the precise ligand(s) recognized by SLTs on murine macrophages is known. However, given the paucity of Gb_3 in macrophage membranes, the data presented here raise the intriguing possibility that SLTs may interact with receptors other than Gb_3 to produce a reproducible and quantifiable outcome, i.e., cytokine production. In addition to our studies, Cohen et al. (13) used fluorescence-activated cell sorter analysis to show that purified SLT-I bound to human tonsillar T lymphocytes and Jurkat (human T-lymphoblast) cells although Gb_3 was undetectable in the membranes of these cells. Although the functional significance of SLT-I binding to T lymphocytes was not addressed, cell viability was not affected by toxin binding. Licastro et al. (31) recently noted that the plant toxin ricin, which shares the same *N*-glycosidase activity as the A subunits of Shiga toxin and SLTs, elicited TNF-α and IL-1β production from human peripheral blood monocytes, suggesting that some degree of toxin enzymatic activity may be necessary to induce cytokine expression. Experiments designed to examine whether mutations introduced into the conserved amino acid sequences defining the active enzymatic sites of SLTs will affect cytokine-inducing capabilities are under way.

The responsiveness of murine cells to a variety of endotoxic activities (e.g., macrophage production of TNF-α, splenocyte proliferation in response to LPS, anti-hapten antibody production in response to hapten-LPS conjugates) is under genetic control (reviewed in reference 60). C3H/HeJ mice differ from the syngeneic C3H/HeN strain at a locus on chromosome 4 designated *lps*, which renders the C3H/HeJ animals specifically hyporesponsive to the endotoxic activities of some forms of the LPS molecule. The precise nature of the mutation responsible for the LPS-hyporesponsive phenotype remains to be elucidated, but a number of studies have supported the concept that the *lps* defect probably involves an alteration in transmembrane signaling in response to LPS binding at the membrane. We show here that, unlike the phenomenon of LPS hyporesponsiveness, TNF activity was consistently induced by purified SLT-I from C3H/HeJ macrophages, although the levels of TNF produced were reduced compared with the activity induced by SLT-I by using syngeneic LPS responder cells. Cytokine production induced by purified SLT preparations was probably not due to minute quantities of contaminating LPS since polymyxin B failed to reduce TNF-α production by C3H/HeN macrophages stimulated with SLT-I. Taken together, these findings suggest that LPS and SLTs may utilize different pathways of transmembrane signaling to induce TNF expression. In support of the concept of alternative signaling pathways leading to cytokine production, Bazzoni et al. (6) recently demonstrated that exposure to UV light induced the expression of TNF mRNA and protein from both C3H/HeN and C3H/HeJ macrophages. Interestingly, by using purified SLT-I as a probe for the detection of Gb_3, we noted differences in Gb_3 levels in C3H/HeN and C3H/HeJ macrophages. Earlier studies using galactose oxidase labeling and thin-layer chromatography techniques on gangliosides and neutral glycolipids from LPS-responsive and LPS-hyporesponsive B lymphocytes demonstrated minor quantitative differences and major surface accessibility differences in glycolipids between the two strains (12, 63). Differences in glycolipid expression and orientation in the membrane may, therefore, contribute to the LPS-hyporesponsive phenotype as well as to the reduced levels of TNF

produced by C3H/HeJ macrophages following stimulation with SLTs.

On a per weight basis, SLTs are clearly less efficient with respect to LPS at inducing cytokine expression from murine macrophages: ~10 times more SLT is needed to induce comparable levels of soluble cytokines in macrophage supernatants. With the exception of IL-1α, the kinetics of cytokine induction were also delayed when SLTs were used as stimulants compared with that when stimulation with LPS was used. In keeping with the delayed kinetics of soluble TNF production, we demonstrated that SLT-I induced delayed transcriptional activation of the gene encoding TNF-α in comparison with the LPS transcriptional response. The precise reason for the delay in transcriptional activation is not known; but the delay may reflect the time necessary for SLTs to be internalized or generate a transmembrane signal. It should also be noted that DNA sequences upstream of the TNF-α promoter contain a number of putative transcriptional activator-binding sites, including at least five NF-κB/NF-GMa binding sites, sequences identical to the major histocompatibility complex class II Y box (a member of the CCAAT box family of *cis*-active sites), a purine-rich box, and an Sp1 binding site (reviewed in reference 26). In addition, enhancer core sequences are found in the intron between the third and fourth exons of the murine TNF gene. Given the large number of putative transcriptional regulatory sites, it is not unreasonable to speculate that LPS and SLTs may activate TNF transcription by separate mechanisms. Studies on TNF gene activation also raise an important caveat in extrapolating the data reported here by using murine macrophages to human cells. DNA sequences upstream of the murine and human TNF genes are not highly conserved. For example, an activator protein-2 binding site found upstream of the human TNF gene is absent in the mouse, while the major histocompatibility complex class II Y box is unique to the murine sequence. Therefore, the cytokine response mediated by SLTs in human macrophages may differ from that of murine cells. The delayed expression of IL-6 compared with that of TNF in LPS- or SLT-stimulated cells is consistent with earlier studies showing that TNF-α and IL-1 may augment the production of IL-6. Finally, studies utilizing human peripheral blood monocytes showed that LPS rapidly induced IL-1α mRNA and resulted in the intracellular accumulation of the 31-kDa precursor form of IL-1α (36). However, the release of the 17-kDa form of the molecule from monocytes was significantly delayed, being immunoprecipitated from supernatants ~12 h after stimulation (21). In accordance with these results, we also detected the delayed release of immunoreactive IL-1α from LPS- or SLT-stimulated murine macrophages.

ACKNOWLEDGMENTS

We thank Christina Kerry, Deanna Lane, Eric Silva, and Susan Turcovski for excellent technical assistance. We thank Roderick McCallum for thoughtful suggestions throughout the course of these experiments.

This work was supported by a grant from the Council for Tobacco Research—U.S.A. and by an Enhancing Excellence in Research Grant (186453) from Texas A&M University Health Science Center.

REFERENCES

1. **Akira, S., T. Hirano, T. Taga, and T. Kishimoto.** 1990. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J. **4**:2860–2867.
2. **Bale, J. F., C. Brasher, and R. L. Siegler.** 1980. CNS manifestations of the hemolytic uremic syndrome. Relationship to metabolic alterations and prognosis. Am. J. Dis. Child. **134**:869–872.
3. **Barrett, T. J., M. E. Potter, and N. A. Strockbine.** 1990. Evidence for participation of the macrophage in Shiga-like toxin II-induced lethality in mice. Microb. Pathog. **9**:95–103.
4. **Barrett, T. J., M. E. Potter, and I. K. Wachsmuth.** 1989. Continuous peritoneal infusion of Shiga-like toxin II (SLT II) as a model for SLT II-induced diseases. J. Infect. Dis. **159**:774–777.
5. **Baud, L., J.-P. Oudinet, M. Bens, L. Noe, M.-N. Peraldi, E. Rondeau, J. Etienne, and R. Ardaillou.** 1989. Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. Kidney Int. **35**:1111–1118.
6. **Bazzoni, F., V. Kruys, A. Shakov, C. V. Jongeneel, and B. Beutler.** 1994. Analysis of tumor necrosis factor promoter responses to ultraviolet light. J. Clin. Invest. **93**:56–62.
7. **Beutler, B., N. Krochin, I. W. Milsark, C. Luedke, and A. Cerami.** 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanism of endotoxin resistance. Science **232**:977–980.
8. **Bhakdi, S., M. Muhly, S. Korom, and G. Schmidt.** 1990. Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. J. Clin. Invest. **85**:1746–1753.
9. **Bromander, A., J. Holmgren, and N. Lycke.** 1991. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages *in vitro*. J. Immunol. **146**:2908–2914.
10. **Brown, J. E., P. Echeverria, and A. A. Lindberg.** 1991. Digalactosyl-containing glycolipids as cell surface receptors for Shiga toxin of *Shigella dysenteriae* 1 and related cytotoxins of *Escherichia coli*. Rev. Infect. Dis. **13**(Suppl. 4):S298–S303.
11. **Caput, D., B. Beutler, K. Hartog, R. Thayer, S. Brown-Shimer, and A. Cerami.** 1986. Identification of a common nucleotide sequence in the 3′-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA **83**:1670–1674.
12. **Chaby, R., M.-J. Morelec, D. Ensergueix, and R. Girard.** 1986. Membrane glycolipid and phospholipid composition of lipopolysaccharide-responsive and -nonresponsive murine B lymphocytes. Infect. Immun. **52**:777–785.
13. **Cohen, A., V. Madrid-Marina, Z. Estrov, M. H. Freedman, C. A. Lingwood, and H.-M. Dosch.** 1990. Expression of glycolipid receptors to Shiga-like toxin on human B-lymphocytes: a mechanism for the failure of long-lived antibody response to dysenteric disease. Int. Immunol. **2**:1–8.
14. **Dinarello, C. A.** 1991. Interleukin-1, p. 47–82. *In* A. Thomson (ed.), The cytokine handbook. Academic Press Ltd., London.
15. **Federal Register.** 1986. Recombinant DNA research guidelines. Fed. Regist. **51**:16972.
16. **Flegel, W. A., F. Müller, W. Däubener, H.-G. Fischer, U. Hadding, and H. Northoff.** 1991. Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. Infect. Immun. **59**:3659–3666.
17. **Fontaine, A., J. Arondel, and P. J. Sansonetti.** 1988. Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox$^-$ mutant of *Shigella dysenteriae* 1. Infect. Immun. **56**:3099–3109.
18. **Fort, P., L. Marty, M. Piechaczyck, S. El Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde 3-phosphate dehydrogenase multigenic family. Nucleic Acids Res. **13**:1431–1442.
19. **Hanna, P. C., D. Acosta, and R. J. Collier.** 1993. On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. USA **90**:10198–10201.
20. **Harel, Y., M. Silva, B. Giroir, A. Weinberg, T. B. Cleary, and B. Beutler.** 1993. A reporter transgene indicates renal-specific induction of tumor necrosis factor (TNF) by Shiga-like toxin. Possible involvement of TNF in hemolytic uremic syndrome. J. Clin. Invest. **92**:2110–2116.
21. **Hazuda, D. J., J. C. Lee, and P. R. Young.** 1988. The kinetics of interleukin 1 secretion from activated monocytes. Differences between interleukin 1α and interleukin 1β. J. Biol. Chem. **263**:8473–8479.
22. **Hill, M. R., and R. E. McCallum.** 1992. Identification of tumor necrosis factor as a transcriptional regulator of the phosphoenolpyruvate carboxykinase gene following endotoxin treatment of mice. Infect. Immun. **60**:4040–4050.

23. **Ikejima, T., S. Okusawa, J. W. M. van der Meer, and C. A. Dinarello.** 1988. Induction by toxic-shock syndrome toxin-1 of a circulating tumor necrosis factor-like substance in rabbits and of immunoreactive tumor necrosis factor and interleukin-1 from human mononuclear cells. J. Infect. Dis. **158:**1017–1025.
24. **Jacewicz, M., H. A. Feldman, A. Donohue-Rolfe, K. A. Balasubramanian, and G. T. Keusch.** 1989. Pathogenesis of *Shigella* diarrhea. XIV. Analysis of Shiga toxin receptors on cloned HeLa cells. J. Infect. Dis. **159:**881–889.
25. **Jevnikar, A. M., D. C. Brennan, G. G. Singer, J. E. Heng, W. Maslinski, R. P. Wuthrich, L. H. Glimcher, and V. E. Rubin Kelley.** 1991. Stimulated kidney tubular epithelial cells express membrane associated and secreted TNFα. Kidney Int. **40:**203–211.
26. **Jongeneel, C. V.** 1992. The TNF and lymphotoxin promoters, p. 539–559. *In* B. Beutler (ed.), Tumor necrosis factors: the molecules and their emerging role in medicine. Raven Press, New York.
27. **Karmali, M. A., M. Petric, C. Lim, R. Cheung, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
28. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18:**775–779.
29. **Kornbluth, R. S., and T. S. Edgington.** 1986. Tumor necrosis factor production by human monocytes is a regulated event: induction of TNF-α-mediated cellular cytotoxicity by endotoxin. J. Immunol. **137:**2585–2591.
30. **Koster, F., J. Levin, L. Walker, K. S. K. Tung, R. H. Gilman, M. Rahaman, M. A. Majid, S. Islam, and R. C. Williams.** 1978. Hemolytic-uremic syndrome after shigellosis. Relation to endotoxemia and circulating immune complexes. N. Engl. J. Med. **298:**927–933.
31. **Licastro, F., M. C. Morini, A. Bolognesi, and F. Stirpe.** 1993. Ricin induces the production of tumour necrosis factor-α and interleukin-1β by human peripheral blood mononuclear cells. Biochem. J. **294:**517–520.
32. **Lieberman, A. P., P. M. Pitha, H. S. Shin, and M. L. Shin.** 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc. Natl. Acad. Sci. USA **86:**6348–6352.
33. **Louise, C. B., and T. G. Obrig.** 1991. Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1β, and tumor necrosis factor alpha on human vascular endothelial cells in vitro. Infect. Immun. **59:**4173–4179.
34. **Louise, C. B., and T. G. Obrig.** 1992. Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro. Infect. Immun. **60:**1536–1543.
35. **Maloney, M., and C. A. Lingwood.** 1993. Interaction of verotoxins with glycosphingolipids. Trends Glycosci. Glycotechnol. **5:**23–31.
36. **Matsushima, K., M. Taguchi, E. J. Kovacs, H. A. Young, and J. J. Oppenheim.** 1986. Intracellular localization of human monocyte associated interleukin 1 (IL 1) activity and release of biologically active IL 1 from monocytes by trypsin and plasmin. J. Immunol. **136:**2883–2891.
37. **Misfeldt, M. L., P. K. Legaard, S. E. Howell, M. H. Fornella, and R. D. LeGrand.** 1990. Induction of interleukin-1 from murine peritoneal macrophages by *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **58:**978–982.
38. **Morrison, D. C., and J. L. Ryan.** 1987. Endotoxins and disease mechanisms. Annu. Rev. Med. **38:**417–432.
39. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
40. **National Institutes of Health.** 1985. Guide for the care and use of laboratory animals. NIH publication no. 85–23. National Institutes of Health, Bethesda, Md.
41. **Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like structural genes from a toxin converting phage of *Escherichia coli*. Science **230:**179–181.
42. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**67–94.
43. **Obrig, T. G., P. J. DelVecchio, J. E. Brown, T. P. Moran, B. M. Rowland, T. K. Judge, and S. W. Rothman.** 1988. Direct cytotoxic action of Shiga toxin on human vascular endothelial cells. Infect. Immun. **56:**2373–2378.
44. **Parsonnet, J., and Z. A. Gillis.** 1988. Production of tumor necrosis factor by human monocytes in response to toxic-shock-syndrome toxin-1. J. Infect. Dis. **158:**1026–1033.
45. **Raetz, C. R. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan.** 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. FASEB J. **5:**2652–2660.
46. **Remuzzi, G.** 1987. HUS and TTP: variable expression of a single entity. Kidney Int. **32:**292–308.
47. **Richardson, S. E., M. A. Karmali, L. E. Becker, and C. R. Smith.** 1988. The histopathology of the hemolytic uremic syndrome (HUS) associated with verocytotoxin-producing *Escherichia coli* (VTEC) infections. Hum. Pathol. **19:**1102–1108.
48. **Richardson, S. E., T. A. Rotman, V. Jay, C. R. Smith, L. E. Becker, M. Petric, N. F. Olivieri, and M. A. Karmali.** 1992. Experimental verocytotoxemia in rabbits. Infect. Immun. **60:**4154–4167.
49. **Samuel, J. E., L. P. Perera, S. Ward, A. D. O'Brien, V. Ginsberg, and H. C. Krivan.** 1990. Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect. Immun. **58:**611–618.
50. **Sandvig, K., O. Garred, K. Prydz, J. V. Kozlov, S. H. Hansen, and B. van Deurs.** 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature (London) **358:**510–512.
51. **Santucci, L. A., P. L. Gutierrez, and D. J. Silverman.** 1992. *Rickettsia rickettsii* induces superoxide radical and superoxide dismutase in human endothelial cells. Infect. Immun. **60:**5113–5118.
52. **Strockbine, N. A., L. R. M. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50:**695–700.
53. **Sung, L. M., M. P. Jackson, A. D. O'Brien, and R. K. Holmes.** 1990. Transcription of the Shiga-like toxin type II and Shiga-like toxin type II variant operons of *Escherichia coli*. J. Bacteriol. **172:**6386–6395.
54. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
55. **Tesh, V. L., J. A. Burris, J. W. Owens, V. M. Gordon, E. A. Wadolkowski, A. D. O'Brien, and J. E. Samuel.** 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect. Immun. **61:**3392–3402.
56. **Tesh, V. L., and A. D. O'Brien.** 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. Mol. Microbiol. **5:**1817–1822.
57. **Tesh, V. L., and J. E. Samuel.** 1993. Immunomodulatory properties of purified Shiga toxin, abstr. B-93, p. 42. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
58. **Tesh, V. L., J. E. Samuel, L. P. Perera, J. B. Sharefkin, and A. D. O'Brien.** 1991. Evaluation of the role of Shiga and Shiga-like toxins in mediating direct damage to human vascular endothelial cells. J. Infect. Dis. **164:**344–352.
59. **Van der Kar, N. C. A. J., L. A. H. Monnens, M. A. Karmali, V. W. M. van Hinsbergh.** 1992. Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood **80:**2755–2764.
60. **Vogel, S. N.** 1992. The *lps* gene. Insights into the genetic and molecular basis of LPS responsiveness and macrophage differentiation, p. 485–513. *In* B. Beutler (ed.), Tumor necrosis factors: the molecules and their emerging role in medicine. Raven Press, New York.
61. **Wadolkowski, E. A., L. M. Sung, J. A. Burris, J. E. Samuel, and A. D. O'Brien.** 1990. Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin type II. Infect. Immun. **58:**3959–3965.
62. **Wallenberg, G. K., L. E. DeForge, G. Bolgos, and D. G. Remick.**

1993. Differential expression of tumor necrosis factor and interleukin-6 by peritoneal macrophages in vivo and in culture. Am. J. Pathol. **143:**1121–1130.

63. **Yohe, H. C., C. S. Berenson, C. L. Cuny, and J. L. Ryan.** 1990. Altered B-lymphocyte membrane architecture indicated by ganglioside accessibility in C3H/HeJ mice. Infect. Immun. **58:**2888–2894.
64. **Zhong, W. W., P. A. Burke, A. T. Hand, M. J. Walsh, L. A. Hughes, and R. A. Forse.** 1993. Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. Arch. Surg. **128:**158–164.
65. **Zoja, C., D. Corna, C. Farina, G. Sacchi, C. Lingwood, M. P. Doyle, V. V. Padhye, M. Abbate, and G. Remuzzi.** 1992. Verotoxin glycolipid receptors determine the localization of microangiopathic process in rabbits given verotoxin-1. J. Lab. Clin. Med. **120:** 229–238.
66. **Zuckerman, S. H., J. Shellhaas, and L. D. Butler.** 1989. Differential regulation of lipopolysaccharide-induced interleukin-1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. Eur. J. Immunol. **19:**301–305.